Clin. exp. Immunol. (1990) **79**, 315–321

REVIEW

Bispecific antibody: a tool for diagnosis and treatment of disease

S. SONGSIVILAI & P. J. LACHMANN *Molecular Immunopathology Unit, Medical Research Council Centre and University of Cambridge Clinical School, Cambridge, England*

(Accepted for publication 14 November 1989)

SUMMARY

Antibodies with two distinct binding specificities have great potential for a wide range of clinical applications as targeting agents for *in vitro* and *in vivo* immunodiagnosis and therapy, and for improving immunoassays. They have shown great promise for targeting cytotoxic effector cells, delivering radionuclides, toxins or cytotoxic drugs to specific targets, particularly tumour cells. We discuss potential applications of bispecific antibodies, the theoretical basis and problems associated with their production and purification, cell fusion and chemical conjugation techniques, and propose a new manufacturing strategy by genetic engineering. This approach has enormous potential applications for producing tailor-made bispecific antibodies, and will enable widespread clinical uses of these antibodies both for diagnostic purposes and therapy.

Keywords bispecific antibodies protein engineering immunotherapy immunoassay

INTRODUCTION

In the first decade of this century Paul Ehrlich proposed the idea of using 'bodies which possess a particular affinity for a certain organ . . . as a carrier by which to bring therapeutically active groups to the organ in question' (Ehrlich, 1906). Since then immunologists have tried to use immunoglobulins for specific diagnosis and treatment of cancer, largely by polyclonal antibodies. The results have been encouraging but, as yet, largely unsuccessful. The introduction of hybridoma technology for producing monoclonal antibodies (Kohler & Milstein, 1975) has revolutionized almost every field of modern medicine, including tumour targeting. Monoclonal antibodies seem to be the ideal 'magic bullets' for specific targeting of tumours.

Because of the inadequate ability of antibodies to be therapeutically or diagnostically efficient on their own, efforts have been made to increase the efficiency of monoclonal antibodies by attaching them to various agents such as bacterial or plant toxins, radionuclides and cytotoxic drugs. Research in the past has been concentrated on using reagents derived mainly from the direct conjugation of antibodies to the effector compounds, which has been accomplished by several distinct methods of covalent coupling (reviewed by Ghose & Blair, 1987; Blakey *et al.*, 1988). These 'immunoconjugates' have now been used for diagnosis and therapy (reviewed by Goldenberg, 1989).

Direct coupling of antibodies to effector compounds has some major disadvantages. Chemical manipulation can inactivate antibody binding sites as well as cause crucial alterations in the effector agents (Hurwitz *et al.*, 1975), thus decreasing the efficiency of the immunoconjugates. Problems may also arise if the covalent bonds between the carrier antibody and the effector compound needs to be broken for full biological action since such bonds may not be easily split (Raso & Griffin, 1981). Another major disadvantage is the non-specific interaction of the Fc domain of an immunoconjugate with its receptor on cells of the reticuloendothelial system, resulting in the undesired accumulation of antibody in some organs, notably the liver and spleen. This high background of labelled antibodies affects the sensitivity of tumour imaging, and also leads to non-specific destruction of cells and organs. The use of Fab fragments, instead of whole antibody molecules, overcomes some of the difficulties but fails to resolve this problem completely (Goldenberg, 1988).

There is evidence that antibodies which bind non-specifically, probably via their Fc portion, are cleared from the body faster than those that bind specifically to their tumour targets (Henkel *et al.*, 1985; and reviewed by Goldenberg, 1988). This has led to the development of an alternative approach for tumour targeting in which instead of coupling effector compounds directly to antibody, a multi-stage delivery system has been employed. Bispecific antibodies which have two different antigen-specific binding sites, one for tumour-associated antigen (target binding arm) and the other for the effector compounds (effector binding arm), have been developed. The specifically designed bispecific monoclonal antibody is firstly targeted to the tumour site by its tumour specificity. After allowing a suitable period of time for the non-specifically bound antibody to be cleared, the effector compound, which is recognized by the second specificity of the targeted antibody, is then injected separately, leading to its specific localization to the tumour. This

Correspondence: Dr Sirirurg Songsivilai, Molecular Immunopathology Unit, MRC Centre, Cambridge CB2 2QH, England

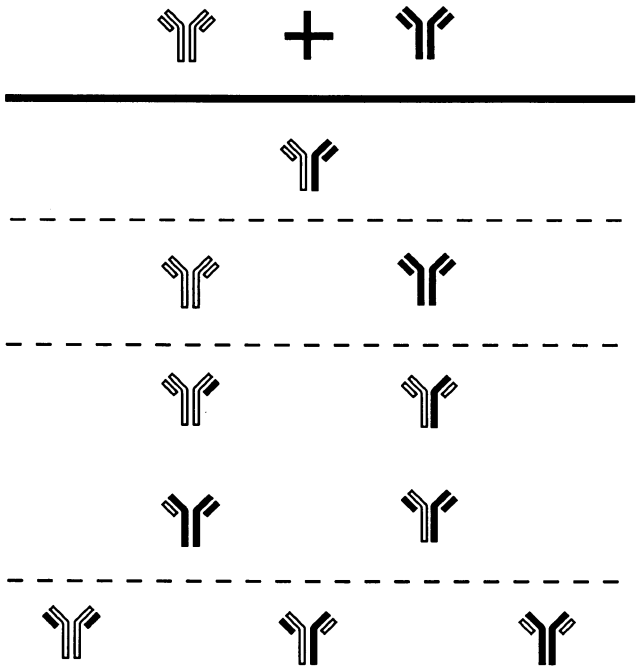

Fig. 1. Diagrammatic representation of ten different species of immunoglobulin molecules secreted by a hybrid hybridoma cell as a result of random association of the heavy and light chains from both parental hybridomas.

system may minimize the toxicity in therapy and maximize the quality of tumour imaging. These bispecific antibodies also have many potential uses ranging from immunodiagnosis to targeted delivery of toxic substances to tumours. They have been studied for targeting effector cells or toxins to tumours and initial results look very promising.

Bispecific antibodies have several advantages over the conventional immunoconjugates. The chemical damage to toxins or cytotoxic drugs during the coupling process is avoided and these compounds are released to specific targets without splitting of covalent bonds. Non-specific toxicity may also be reduced. Bispecific antibodies are structurally bivalent but functionally univalent for each antigen-binding site. The mechanism whereby target cells escape killing by redistributing and eventually losing the antigen–antibody complexes from their surface, known as antigenic modulation (Gordon, Robinson & Stevenson, 1981; Gordon & Stevenson, 1981; Cobbold & Waldmann, 1984), may also be minimized due to the monovalency of the bispecific antibody (Glennie *et al.*, 1988).

PRODUCTION OF BISPECIFIC ANTIBODIES

Bispecific antibodies are produced mainly by two methods: fusion of two different hybridoma cell lines, or chemically linking two antibody molecules or their derivatives.

Fusion of hybridomas

Normal plasma cells secrete only one set of heavy and light chains of immunoglobulin as a result of allelic exclusion. Hybridoma cell lines producing monoclonal antibodies derived from the fusion of the 'non-secreting' parental myeloma cell lines with immune cells, thus also secrete one set of immunoglobulins. However, fusion of two myeloma clones, each secreting different immunoglobulins, resulted in the co-dominant expression of both parental immunoglobulin genes and two sets of heavy and light chains were found to be secreted (Cotton & Milstein, 1973; Schwaber & Cohen, 1974). The assembly of immunoglobulin molecules thus allows the formation of both parental immunoglobulins, and also the hybrid molecules. A number of molecular species can be predicted theoretically (Suresh, Cuello & Milstein, 1986a). If total random association of heavy and light chains occurs, ten different combinations of immunoglobulin molecules are generated; however, only one has the desired bispecific activity (Fig. 1).

This approach has been used to produce several bispecific antibodies. Fusions have been done between pairs of hybridoma cell lines secreting two existing monoclonal antibodies (Suresh *et al.*, 1986b; Tiebout *et al.*, 1987; Urnovitz *et al.*, 1988), or between hybridoma cell lines and immune spleen cells (Kohler & Milstein, 1975; Milstein & Cuello, 1983; Webb *et al.*, 1985). The former is preferable since the specificity of the resulting bispecific antibody should be more predictable. The quality and affinity of bispecific antibodies derived from the latter remains a matter of chance and depends on the contribution of spleen cell partner, requiring the characterization of the specificity of the hybrid antibodies. The main advantage of the fusion technique is that the resulting bispecific antibodies are synthesized, assembled, and secreted by the same process as that of the native immunoglobulin. Their stability, both *in vitro* and *in vivo*, and pharmacokinetics are theoretically comparable to those of the normal antibodies. Once the 'hybrid hybridoma' cell lines are obtained, they will serve as the machines to produce endless amounts of antibody in the same way as normal hybridomas.

There are some disadvantages: difficulty of fusing hybrid hybridoma cells, stability of the resulting cell lines, low yields, and difficulty in purificaiton of the bispecific molecules. Cell fusion is labour-intensive, time consuming, and may not always succeed with the hybridoma pairs of choice. Not all hybridoma cell lines exhibit good fusion performance (Suresh *et al.*, 1986a; Songsivilai, unpublished data). The lack of an easy method for selecting the hybrid hybridoma cells results from the fact that most parental hybridomas are derived from the fusion of HAT-sensitive myeloma fusion partners and immune spleen cells, and are thus HAT resistant. Several approaches have been developed. Parental hybridoma cell lines were reverted to HAT-sensitive by selecting mutants that lack hypoxanthine–guanine phosphoribosyltransferase (HGPRT) marker in the medium containing 8-azaguanine or 6-thioguanine, then fused with the immune spleen cells (Milstein & Cuello, 1983). Alternatively hybridoma cells lacking other markers such as thymidine kinase or adenosine phosphoribosyltransferase may also be selected in the medium containing bromodeoxyuridine or 6-chloropurine, respectively. Two hybridoma cell lines lacking two independent markers have also been fused and the resulting hybrid cells selected in simple HAT-medium (Wong & Colvin, 1987; Urnovitz *et al.*, 1988). Other selectable markers, such as the resistance to neomycin, methotrexate or actinomycin D, may also be introduced into cells by means of gene transfection (Lanzavecchia & Scheidegger, 1987; Chervonsky *et al.*, 1988; De Lau *et al.*, 1989). An alternative approach is to use two distinct site-specific irreversible inhibitors of protein synthesis, such as emetine, actinomycin D, or iodoacetamide, to inhibit two independent metabolic pathways of each of the two parental cell

lines. Fused cells apparently survived by complementing each other (Suresh *et al.*, 1986b). This method has been used in combination with HAT selection by fusing the HAT-sensitive hybridoma cell lines with the chemically treated hybridomas (Suresh *et al.*, 1986a; Clark & Waldmann, 1987). Alternatively, hybrid hybridomas may also be selected by a fluorescent-activated cell sorter (FACS) without relying on drug selection (Karawajew *et al.*, 1987; Koolwijk *et al.*, 1988).

If the rate of production of the two pairs of heavy and light chain is the same and the association of heavy and light chains shows no homologous or heterologous preference (i.e., the association is totally random), then the yield of the desired bispecific antibody is 12·5% (2/16) of the total immunoglobulin secreted by the hybrid cell (Staerz & Bevan, 1986). Prediction of the yields of bispecific antibody is difficult, since preferential association may occur, and thus yields can range from 0 to 50%. The ideal condition should be an absolute homologous preference in heavy–light chain pairing and random pairing of heavy chains, then the yield of the desired bispecific antibody will be 50% of total immunoglobulin (Corvalan & Smith, 1987). However, the total absence of association between heavy chains of different class or subclass, such as IgM : IgG or IgA : IgG, may prevent the formation of bispecific molecules (Zimmerman & Grey, 1971; Takahashi & Fuller, 1988; Urnovitz *et al.*, 1988).

The stability of hybrid hybridoma cell lines should be questioned. Chromosomes of these hybrid cells are polyploid, approximately equal to the sum of the chromosomes of both parental hybridomas (Kohler & Milstein, 1975; Koolwijk *et al.*, 1988) and therefore unstable. This was shown in early work on hybridoma fusion in which the first myeloma fusion partner secreted murine IgG1, resulting in a hybridoma secreting mixed molecules, which at that time was considered to be a disadvantage. Due to the instability of their chromosomes, mutants which lost immunoglobulin genes from the parental myeloma were easily cloned and monoclonal antibody-secreting cells selected. For the continuing production of bispecific antibodies, the 'hybrid hybridoma' cell lines may require frequent cloning to maintain the presence of both sets of heavy and light chains.

Since a total of ten species of molecules are formed, isolation of the bispecific antibody can be difficult. Purification has been achieved by isoelectric focusing (Wong & Colvin, 1987), hydroxylapatite chromatography (Staerz & Bevan, 1986; Karawajew *et al.*, 1987), ion-exchange chromatography (Suresh *et al.*, 1986b), or double affinity chromatography (Corvalan & Smith, 1987).

Chemical linking of antibody molecules or their derivatives

The technique for producing bispecific antibodies by chemical manipulation was pioneered by Nisonoff & Rivers (1961). It does not require cell fusion, the desired bispecific antibodies can be made more quickly and the products are also comparatively easy to purify (Karpovsky *et al.*, 1984; Glennie *et al.*, 1987).

Chemical coupling can be achieved in two ways: direct coupling of the whole antibody molecules or their derivatives, and dissociation and reassociation of heterologous immunoglobulin. The latter requires chemical manipulation to dissociate immunoglobulins into half molecules without damaging the antigen binding sites, then to reform the disulphide bonds linking the heavy chains without allowing any interfering side reactions such as the formation of intrachain or mismatch disulphide bonds. The stability of the bond is in doubt since it may be cleaved *in vivo*.

Several alternative conjugation techniques have been employed (Karpovsky *et al.*, 1984; Brennan, Davison & Paulus, 1985; Paulus, 1985; Liu *et al.*, 1985; Lansdorp *et al.*, 1986; Glennie *et al.*, 1987). In one study, the yield of heteroconjugate derived by the coupling using hetero-bifunctional linker, SPDP, was about 5% of the initial monoclonal antibody used for conjugation and its activity was partially lost 20 days after coupling (Canevari *et al.*, 1988). Fab fragments have also been used instead of the whole immunoglobulin molecules; for example, they can be linked by thioether bonds using *o*-phenylenedimaleimide. The yield and stability of these molecules have been claimed to be better than the disulphide-linked molecules (Glennie *et al.*, 1987, 1988).

Chemical heteroconjugates of monoclonal antibodies have different physical and biological properties from native immunoglobulin molecules. Chemical manipulations frequently disturb the biological activity of antibody by alteration of the antigen binding sites (Webb *et al.*, 1985). The 'heteroconjugates' derived by direct coupling of antibody molecules may have difficulty penetrating the target site due to their size. However, the lack of Fc of the $F(ab')_2$ heteroconjugates and their small size will also shorten the plasma half life; their stability *in vitro* and *in vivo*, and their pharmacokinetics, have yet to be investigated.

ENGINEERING OF BISPECIFIC ANTIBODIES

The clinical use of monoclonal antibodies, mostly derived from murine cells, faces the problem of anti-globulin response. This limits their application especially when repeated injections are required. In addition, a severe hypersensitivity reaction may occur. Xenogeneic antibodies are also not well fitted to destroy cells *in vivo* because the complement and cellular effectors (K cells and phagocytes) are not recruited efficiently (Stevenson *et al.*, 1988). In a recent development, genetically engineered chimaeric human/mouse monoclonal antibodies have been developed by replacing the Fc region of the murine immunoglobulin molecule with the human constant region (Morrison *et al.*, 1984; Boulianne, Hozumi & Shulman, 1984; Neuberger *et al.*, 1985). The framework regions of variable domains of murine immunoglobulin have also been replaced by their human counterpart, or vice versa (Jones *et al.*, 1986). These chimaeric antibodies have been shown to be less immunogenic than the native murine antibodies. Genetic engineering has also been used to produce tailor-made antibodies with special properties, such as single-chain Fv and single-domain antibodies (Huston *et al.*, 1988; Bird *et al.*, 1988; Ward *et al.*, 1989).

Bispecific antibodies have been produced by introducing two sets of immunoglobulin heavy and light chain genes into myeloma cells or by transfecting a set of heavy and light chain genes into secreting hybridoma or transfectoma cell lines. Chimaeric bispecific antibodies have been identified using both techniques (Songsivilai, Clissold & Lachmann, 1989; Johnson *et al.*, 1989). Since only the variable regions are derived from the parent hybridomas, the constant regions of the two heavy chains of chimaeric immunoglobulins can be selected to allow total random association of heavy chains for the best yield of bispecific antibodies. Bispecific antibodies that cannot be produced due to the inability of parental heavy chains to form

stable molecules can also be engineered by replacing the heavy chain with a suitable class or subclass. A matched set of 'mixed isotype' bispecific antibodies with the same specificities may also be produced. For some applications where polyvalent, rather than monovalent, bispecific antibodies are preferred (Laky *et al.*, 1987; Urnovitz *et al.*, 1988), dimeric IgA or pentameric IgM bispecific or multi-specific monoclonal antibodies may be constructed. Transfectomas secreting bispecific antibodies will have a smaller number, and less complexity of chromosomes compared with hybrid hybridomas from cell fusion. Since only small chimaeric genes are introduced and then integrated into host genome, the resulting transfectoma cell lines should be more stable.

Antibody engineering will have enormous applications for producing tailor-made bispecific antibodies. For example, preferential homologous heavy–light chain association may theoretically be driven by selecting suitable pairs of heavy and light chains. Genetically engineered single-peptide bispecific antibodies (such as VH1-VL1–VH2-VL2) may also be constructed using peptide linkers between each variable domain. This construct will allow 100% yield of bispecific molecules. Other expression systems such as *Escherichia coli* may increase the yield of the engineered bispecific antibodies.

APPLICATIONS OF BISPECIFIC ANTIBODIES

Immunoassays

The effector binding arm can be designed to have specificity for marker enzymes or other indicator systems. The anti-target–anti-peroxidase bispecific antibodies which have been used in immunohistochemistry have led to improvements in sensitivity, signal-to-noise ratio, and simplification of staining procedures with preservation of fine ultrastructural detail (Milstein & Cuello, 1983; Suresh *et al.*, 1986b; Ribeiro da Silva, Tagari & Cuello, 1989). These reagents may also simplify or improve diagnostic techniques, such as in single-step immunoassays and other assay systems (Leong, Milstein & Pannell, 1986; Karawajew *et al.*, 1988; Takahashi & Fuller, 1988; Tada, Toyoda & Iwasa, 1989).

Tumour targeting

The use of bispecific antibodies for immunodiagnosis and therapy has shown some encouraging results. They have been used for delivering effector substances such as toxins (Corvalan *et al.*, 1987; Webb *et al.*, 1989) and cytotoxic drugs to tumours, and some are now in clinical trials (Stickney, Slater & Frincke, 1989).

Cross-linking of cellular antigens and focusing of effector cells

Many efforts have been made to use bispecific antibodies to focus cytotoxic effector cell response to tumour targets. This system has been studied either *in vitro* and *in vivo*, in animal and human models, using both the heteroconjugates and the hybrid bispecific antibodies. Several effector binding specificities were used. These included antibodies to Fc receptor (Karpovsky *et al.*, 1984; Shen *et al.*, 1986), T cell receptor/CD3 complexes (Liu *et al.*, 1985; Perez *et al.*, 1985; Staerz, Kanagawa & Bevan, 1985; Jung *et al.*, 1986; Barr *et al.*, 1987; Lanzavecchia & Scheidegger, 1987; Clark & Waldmann, 1987; Rammensee *et al.*, 1987; Vyakarnam *et al.*, 1988; van Dijk *et al.*, 1989; Roosnek & Lanzavecchia, 1989), and CD2 molecules (Goedegebuure *et al.*, 1989). Bispecific antibodies which bind to target cells can activate effector cells, and cross-link the targets to the effector cells. Lysis of virus-infected target cells has also been observed (Staerz, Yewdell & Bevan, 1987). The use of bispecific antibodies may not simply serve to glue the targets and effector cells together, but may also trigger the cytolytic process (Karpovsky *et al.*, 1984). Cytotoxicity has been shown not to be due to bystander lysis, since direct contact between effector and target cells is required (Barr *et al.*, 1987; Lanzavecchia & Scheidegger, 1987).

Most of the experiments on effector cell targeting were performed using homologous effector cell populations, such as cloned T cells. The mechanisms of cytotoxicity *in vivo* may be different and may involve several killing systems. Destruction of the putative effector cells (Lanzavecchia & Scheidegger, 1987), possibly due to the fact that the bispecific antibody-bound effector cells may themselves serve as targets for antibody-dependent cell-mediated cytotoxicity, has been observed. It seems that mixed isotype bispecific antibodies, such as rat IgG2b–IgG2c, which can mediate cytotoxicity of target cells by non-ADCC mechanisms, may minimize this problem (Clark & Waldmann, 1987).

Specific delivery of effector compounds to targets

Targeting toxic compounds to tumours has been investigated by using anti-CEA–anti-vinca alkaloid hybrid bispecific antibodies (Corvalan *et al.*, 1987). Radiolabelled vinblastine sulphate was localized at the tumour sites when injected with or after the bispecific antibodies. The background radiation in other organs such as liver and spleen was low compared with the radiolabelled drug alone. Therapeutic data produced in the *in vivo* mouse xenografted model indicated that this method was more effective in suppressing tumour growth than the vinca alkaloids when given as free drug (Corvalan, Smith & Gore, 1988). A study using anti-idiotype–anti-saporin heteroconjugates for treatment of lymphoma also showed encouraging results (Glennie *et al.*, 1988). Clinical studies using heteroconjugate bispecific $F(ab)_2$ anti-CEA–anti-BLEDTA IV, an In-111 benzyl EDTA derivative of cobalt bleomycin, injected into patients with colon cancer 24–120 hours before the injection of ^{111}In BLEDTA, showed good tumour targeting with low uptake by liver and bone marrow (Stickney *et al.*, 1989).

A multi-stage delivery system using bispecific antibodies may have a disadvantage since its effectiveness relies on the two antigen–antibody interactions, between two arms of bispecific antibodies and both target and effector molecules. This potential problem may be overcome by the use of high-affinity bispecific monoclonal antibodies. The bispecific antibody must also be accessible to the effector molecules on the surface of target cells. This problem may not occur with the monovalent bispecific antibodies.

Bispecific antibodies can be used to distinguish cells that co-express two different surface antigens. Anti-CD3–anti-CD4 and anti-CD3–anti-CD8 bispecific antibodies were shown to promote complement-mediated lysis of target cells that express both the relevant surface antigens 25 to 3125 times more efficiently than those expressing only one of the antigens (Wong & Colvin, 1987).

Several other systems have also been studied, such as direct

targeting of tissue plasminogen activator (tPA) by anti-tPA–anti-fibrin bispecific antibody to enhance thrombolysis (Bode *et al.*, 1989). Anti-interferon (IFN)–anti-target cell heteroconjugates were shown to deliver IFN specifically to target cells and also inhibit their growth *in vitro* (Alkan, Towbin & Hochkeppel, 1988).

PROSPECTS

Bispecific antibodies have potential for a wide range of clinical applications but are still difficult to produce. Areas in which improvement must be made before these methods become widely acceptable include fusion and selection, chemical coupling, and purification.

Universal bispecific antibodies may be produced. Chain-, class-, or isotype-specific antibody may be used as a general target binding arm. This type of construct has been proved effective for indirect effector cell retargeting in an *in vitro* system (Gilliland, Clark & Waldmann, 1988). Hapten-specific antibody may also be used as a general effector binding arm (Songsivilai *et al.*, 1989), allowing the effectiveness of different effector compounds, such as radionuclides, toxins, and cytotoxic drugs attached to carrier hapten to be investigated by the same bispecific antibody. These two approaches may be combined. The universal agents will minimize the need to make bispecific antibodies for a variety of targets and toxic agents.

For the clinical use of bispecific antibodies, human immunoglobulins would be preferred. Human hybrid hybridoma cell lines are difficult to produce (Tiebout *et al.*, 1987) and may have the same theoretical and technical disadvantages as the murine hybridoma cells. Genetic manipulation by introducing sets of chimaeric immunoglobulin genes into myeloma or hybridoma cell lines is an alternative. The new techniques of antibody engineering which may revolutionize the monoclonal antibody technology will be a powerful tool for the production of 'tailor-made' bispecific molecules.

The *in vitro* and *in vivo* properties of the mixed-isotype bispecific antibodies are worth investigating. It may increase our understanding of the effector functions of immunoglobulin, such as Fc receptor binding and complement activation. Alteration of heavy chain pairing may affect the effector functions of monoclonal antibodies which may be of benefit for therapy. A matched set of bispecific antibodies with the same specificity may be of value (Songsivilai *et al.*, 1989).

Antibodies with two distinct binding ends show great promise as targeting agents and for improving immunoassays. When the problems associated with their manufacture have been satisfactorily resolved, they are likely to find widespread clinical applications both for diagnostic purposes and therapy.

ACKNOWLEDGMENTS

We wish to thank Drs K. S. Aulak, P. M. Clissold and M. G. Wing for helpful discussion. S. S. is supported by the Anandhamahidol Foundation and the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

REFERENCES

ALKAN, S.S., TOWBIN, H. & HOCHKEPPEL, H.K. (1988) Enhanced antiproliferative action of interferon targeted by bispecific monoclonal antibodies. *J. Interferon Res.* **8,** 25.

BARR, I.G., MACDONALD, H.R., BUCHEGGER, F. & VON FLIEDNER, V. (1987) Lysis of tumour cells by the retargeting of murine cytolytic T lymphocytes with bispecific antibodies. *Int. J. Cancer*, **40,** 423.

BIRD, R.E., HARDMAN, K.D., JACOBSON, J.W., JOHNSON, S., KAUFMAN, B.M., LEE, S.-M., LEE, T., POPE, S.H., RIORDAN, G.S. & WHITLOW, M. (1988) Single-chain antigen-binding proteins. *Science*, **242,** 423.

BLAKEY, D.C., WAWRZYNCZAK, E.J., WALLACE, P.M. & THORPE, P.E. (1988) Antibody toxin conjugates: a perspective. *Prog. Allergy*, **45,** 50.

BODE, C., RUNGE, M.S., BRANSCOMB, E.E., NEWELL, J.B., MATSUEDA, G.R. & HABER, E. (1989) Antibody-directed fibrinolysis. An antibody specific for both fibrin and tissue plasminogen activator. *J biol. Chem.* **264,** 944.

BOULIANNE, G.L., HOZUMI, N. & SHULMAN, M.J. (1984) Production of functional chimaeric mouse/human antibody. *Nature*, **312,** 643.

BRENNAN, M., DAVISON, P.F. & PAULUS, H. (1985) Preparation of bispecific antibodies by chemical recombination of monoclonal immunoglobulin G1 fragments. *Science*, **229,** 81.

CANEVARI, S., MENARD, S., MEZZANZANICA, D., MIOTTI, S., PUPA, S.M., LANZAVECCHIA, A. & COLNAGHI, M.I. (1988) Anti-ovarian carcinoma anti-T3 heteroconjugates or hybrid antibodies induce tumor cell lysis by cytotoxic T-cells. *Int. J. Cancer*, **2,** (Suppl.) 18.

CHERVONSKY, A.V., FAERMAN, A.I., EVDONINA, L.V., JAZOVA, A.K., KAZAROV, A.R. & GUSSEV, A.I. (1988) A simple metabolic system for selection of hybrid hybridomas (tetradomas) producing bispecific monoclonal antibodies. *Mol. Immunol.* **25,** 913.

CLARK, M.R. & WALDMANN, H. (1987) T-cell killing of target cells induced by hybrid antibodies: comparison of two bispecific monoclonal antibodies. *JNCI*, **79,** 1393.

COBBOLD, S.P. & WALDMANN, H. (1984) Therapeutic potential of monovalent monoclonal antibodies. *Nature*, **308,** 460.

CORVALAN, J.R.F. & SMITH, W. (1987) Construction and characterisation of a hybrid–hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. *Cancer Immunol. Immunother.* **24,** 127.

CORVALAN, J.R.F., SMITH, W. & GORE, V.A. (1988) Tumour therapy with vinca alkaloids targeted by a hybrid-hybrid monoclonal antibody recognising both CEA and vinca alkaloids. *Int. J. Cancer*, **2,** (Suppl.), 22.

CORVALAN, J.R.F., SMITH, W., GORE, V.A. & BRANDON, D.R. (1987) Specific *in vitro* and *in vivo* drug localisation to tumour cells using a hybrid–hybrid monoclonal antibody recognising both carcinoembryonic antigen (CEA) and vinca alkaloids. *Cancer Immunol. Immunother.* **24,** 133.

COTTON, R.G.H. & MILSTEIN, C. (1973) Fusion of two immunoglobulin-producing myeloma cells. *Nature*, **244,** 42.

DE LAU, W.B.M., VAN LOON, A.E., HEIJE, K., VALERIO, D. & BAST, B.J.E.G. (1989) Production of hybrid hybridomas based on HAT^s-$neomycin^r$ double mutants. *J. immunol. Methods*, **117,** 1.

EHRLICH, P. (1906) *Collected Studies on Immunity* Vol 2. p. 442. John Wiley, New York.

GHOSE, T. & BLAIR, A.H. (1987) The design of cytotoxic-agent-antibody conjugates. *Crit. Rev. ther. Drug. Carrier Syst.* **3,** 262.

GILLILAND, L.K., CLARK, M.R. & WALDMANN, H. (1988) Universal bispecific antibody for targeting tumour cells for destruction by cytotoxic T cells. *Proc. natl Acad. Sci. USA*, **85,** 7719.

GLENNIE, M.J., BRENNAND, D.M., BRYDEN, F., MCBRIDE, H.M., STIRPE, F., WORTH, A.T. & STEVENSON, G.T. (1988) Bispecific $F(ab'\gamma)_2$ antibody for the delivery of saporin in the treatment of lymphoma. *J. Immunol.* **141,** 3662.

GLENNIE, M.J., MCBRIDE, H.M., WORTH, A.T. & STEVENSON, G.T. (1987) Preparation and performance of bispecific $F(ab'\gamma)_2$ antibody containing thioether-linked $Fab'\gamma$ fragments. *J. Immunol.* **139,** 2367.

GOEDEGEBUURE, P.S., SEGAL, D.M., BRAAKMAN, E., VREUGDENHIL, R.J., VAN KRIMPEN, B.A., VAN DE GRIEND, R.J. & BOLHUIS, R.L. (1989) Induction of lysis by T cell receptor $\gamma\delta^+$/CD3$^+$ T lymphocytes via CD2 requires triggering via the T11.1 epitope only. *J. Immunol.* **142,** 1797.

GOLDENBERG, D.M. (1988) Targeting of cancer with radiolabelled antibodies: prospects for imaging and therapy. *Arch. Pathol. Lab. Med.* **112,** 580.

GOLDENBERG, D.M. (1989) Targeted cancer treatment. *Immunol. Today*, **10,** 286.

GORDON, J. & STEVENSON, G.T. (1981) Antigenic modulation of lymphocytic surface immunoglobulin yielding resistance to complement-mediated lysis. II. Relationship to redistribution of the antigen. *Immunology*, **42,** 13.

GORDON, J., ROBINSON, D.S.F. & STEVENSON, G.T. (1981) Antigenic modulation of lymphocytic surface immunoglobulin yielding resistance to complement-mediated lysis. I. Characterization with syngeneic and xenogeneic complements. *Immunology*, **42,** 7.

HENKEL, R.D., KENNEDY, R.C., SPARROW, J.T. & DRESSMAN, G.R. (1985) *In vivo* detection of human hepatoma secreting hepatitis B surface antigen in nude mice with radiolabelled monoclonal antibodies that recognised distinct epitopes. *Clin. Immunol. Immunopathol.* **35,** 146.

HURWITZ, E., LEVY, R., MARON, R., WILCHEK, M., ARNON, R. & SELA, M. (1975) The covalent binding of daunomycin and adriamycin to antibodies, with retention of both drug and antibody activities. *Cancer Res.* **35,** 1175.

HUSTON, J.S., LEVINSON, D., MUDGETT-HUNTER, M., TAI, M-S., NOVOTNY, J., MARGOLIES, M.N., RIDGE, R.J., BRUCCOLERI, R.E., HABER, E., CREA, R. & OPPERMANN, H. (1988) Protein engineering of antibody binding sites: recovery of specific activity in an anti-digoxin single-chain Fv analogue produced in *Escherichia coli. Proc. natl. Acad. Sci. USA*, **85,** 5879.

JOHNSON, J., BEIDLER, D., JUE, R.A., UNGER, B. & PHELPS, J. (1989) Biological production of chimeric bifunctional antibodies. In *Seventh International Congress of Immunology*. (Abstract). p. 8. Gustav Fischer Verlag, Stuttgart.

JONES, P.T., DEAR, P.H., FOOTE, J., NEUBERGER, M.S. & WINTER, G. (1986) Replacing the complementarity-determining regions in a human antibody with those from a mouse. *Nature*, **321,** 522.

JUNG, G., HONSIK, C.J., REISFELD, R.A. & MULLER-EBERHARD, H.J. (1986) Activation of human peripheral blood mononuclear cells by anti-T3: killing of tumour target cells coated with anti-target-anti-T3 conjugates. *Proc. natl Acad. Sci. USA*, **83,** 4479.

KARAWAJEW, L., MICHEEL, B., BEHRSING, O. & GAESTEL, M. (1987) Bispecific antibody-producing hybrid hybridomas selected by a fluorescence activated cell sorter. *J. immunol. Methods*, **96,** 265.

KARAWAJEW, L., BEHRSING, O., KAISER, G. & MICHEEL, B. (1988) Production and ELISA application of bispecific monoclonal antibodies against fluorescein isothiocyanate (FITC) and horseradish peroxidase (HRP). *J. immunol. Methods*, **111,** 95.

KARPOVSKY, B., TITUS, J.A., STEPHANY, D.A. & SEGAL, D.M. (1984) Production of target-specific effector cells using hetero-cross-linked aggregates containing anti-target cell and anti-Fcγ receptor antibodies. *J. exp. Med.* **160,** 1686.

KOHLER, G. & MILSTEIN, C. (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*, **256,** 495.

KOOLWIJK, P., ROZEMULLER, E., STAD, R.K., DE LAU, W.B.M. & BAST, B.J.E.G. (1988) Enrichment and selection of hybrid hybridomas by percoll density gradient centrifugation and fluorescent activated cell sorting. *Hybridoma*, **7,** 217.

LAKY, M., MOTA, G., MORARU, I. & GHETIE, V. (1987) Binding and cytotoxic effect of ricin toxin on multivalent hybrid antibody-coated target cells. *Immunol. Lett.* **14,** 127.

LANSDORP, P.M., AALBERSE, R.C., BOS, R., SCHUTTER, W.G. & VAN BRUGGEN, E.F.J. (1986) Cyclic tetramolecular complexes of monoclonal antibodies: a new type of cross-linking reagent. *Eur. J. Immunol.* **16,** 679.

LANZAVECCHIA, A. & SCHEIDEGGER, D. (1987) The use of hybrid hybridomas to target human cytotoxic T lymphocytes. *Eur. J. Immunol.* **17,** 105.

LEONG, M.M.L., MILSTEIN, C. & PANNELL, R. (1986) Luminescent detection method for immunodot, Western, and Southern blots. *J. Histochem. Cytochem.* **34,** 1645.

LIU, M.A., KRANZ, D.M., KURNICK, J.T., BOYLE, L.A., LEVY, R. & EISEN, H.N. (1985) Heteroantibody duplexes target cells for lysis by cytotoxic T lymphocytes. *Proc. natl Acad. Sci. USA*, **82,** 8648.

MILSTEIN, C. & CUELLO, A.C. (1983) Hybrid hybridomas and their use in immunohistochemistry. *Nature*, **305,** 537.

MORRISON, S.L., JOHNSON, M.J., HERZENBERG, L.A. & OI, V.T. (1984) Chimeric human antibody molecules: Mouse antigen-binding domains with human constant region domains. *Proc. natl Acad. Sci. USA*, **81,** 6851.

NEUBERGER, M.S., WILLIAMS, G.T., MITCHELL, E.B., JOUHAL, S.S., FLANAGAN, J.G. & RABBITTS, T.H. (1985) A haptan-specific chimaeric IgE antibody with human physiological effector function. *Nature*, **314,** 268.

NISONOFF, A. & RIVERS, M.M. (1961) Recombination of a mixture of univalent antibody fragments of different specificity. *Arch. Biochem. Biophys.* **93,** 460.

PAULUS, H. (1985) Preparation and biomedical applications of bispecific antibodies. *Behring Inst. Mitt.* **78,** 118.

PEREZ, P., HOFFMANN, R.W., SHAW, S., BLUESTONE, J.A. & SEGAL, D.M. (1985) Specific targeting of cytotoxic T cells by anti-T3 linked to anti-target cell antibody. *Nature*, **316,** 354.

RAMMENSEE, H.G., JULIUS, M.H., NEMAZEE, D., LANGHORNE, J., LAMERS, R. & KOHLER, G. (1987) Targeting cytotoxic T cells to antigen-specific B lymphocytes. *Eur. J. Immunol.* **17,** 433.

RASO, V. & GRIFFIN, T. (1981) Hybrid antibodies with dual specificity for the delivery of ricin to immunoglobulin-bearing targets. *Cancer Res.* **41,** 2073.

RIBEIRO DA SILVA, A., TAGARI, P. & CUELLO, A.C. (1989) Morphological characterisation of substance P-like immunoreactive glomeruli in the superficial dorsal horn of the rat spinal cord and trigeminal subnucleus caudalis: a quantitative study. *J. comp. Neurol.* **281,** 497.

ROOSNEK, E. & LANZAVECCHIA, A. (1989) Triggering T cells by otherwise inert hybrid anti-CD3/antitumor antibodies requires encounter with the specific target cell. *J. exp. Med.* **170,** 297.

SCHWABER, J. & COHEN, E.P. (1974) Pattern of immunoglobulin synthesis and assembly in a human-mouse somatic cell hybrid clone. *Proc. natl. Acad. Sci. USA*, **71,** 2203.

SHEN, L., GUYRE, P.M., ANDERSON, C.L. & FANGER, M.W. (1986) Heteroantibody-mediated cytotoxicity: antibody to the high affinity Fc receptor for IgG mediates cytotoxicity by human monocytes that is enhanced by interferon-γ and is not blocked by human IgG. *J. Immunol.* **137,** 3378.

SONGSIVILAI, S., CLISSOLD, P.M. & LACHMANN, P.J. (1989) A novel strategy for producing chimeric bispecific antibodies by gene transfection. *Biochem. Biophys. Res. Commun.* **164,** 271.

STAERZ, U.D. & BEVAN, M.J. (1986) Hybrid Hybridoma producing a bispecific monoclonal antibody that can focus effector T-cell activity. *Proc. natl. Acad. Sci. USA*, **83,** 1453.

STAERZ, U.D., KANAGAWA, O. & BEVAN, M.J. (1985) Hybrid antibodies can target sites for attack by T cells. *Nature*, **314,** 628.

STAERZ, U.D., YEWDELL, J.W. & BEVAN, M.J. (1987) Hybrid antibody-mediated lysis of virus-infected cells. *Eur. J. Immunol.* **17,** 571.

STEVENSON, G.T., GLENNIE, M.J., HAMBLIN, T.J., LANE, A.C. & STEVENSON, F.K. (1988) Problems and prospects in the use of lymphoma idiotypes as therapeutic targets. *Int. J. Cancer*, **3,** (Suppl.), 9.

STICKNEY, D.R., SLATER, J.B. & FRINCKE, J.M. (1989) Imaging and therapeutic potential of bifunctional antibody (BFA) in colon carcinoma. In *Fourth International Conference on Monoclonal Anti-*

body Immunoconjugates for Cancer, (Abstract). p. 29. UCSD, San Diego.

SURESH, M.R., CUELLO, A.C. & MILSTEIN, C. (1986a) Bispecific monoclonal antibodies from hybrid hybridomas. *Methods Enzymol.* **121,** 210.

SURESH, M.R., CUELLO, A.C. & MILSTEIN, C. (1986b) Advantages of bispecific hybridomas in one-step immunochemistry and immunoassays. *Proc. natl Acad. Sci. USA*, **83,** 7989.

TADA, H., TOYODA, Y. & IWASA, S. (1989) Bispecific antibody-producing hybrid hybridoma and its use in one-step immunoassay for human lymphotoxin. *Hybridoma*, **8,** 73.

TAKAHASHI, M. & FULLER, S.A. (1988) Production of murine hybrid-hybridomas secreting bispecific monoclonal antibodies for use in urease-based immunoassays. *Clin. Chem.* **34,** 1693.

TIEBOUT, R.F., VAN BOXTEL-OOSTERHOF, F., STRICKER, E.A.M. & ZEIJLEMAKER, W.P. (1987) A human hybrid hybridoma. *J. Immunol.* **139,** 3402.

URNOVITZ, H.B., CHANG, Y., SCOTT, M., FLEISCHMAN, J. & LYNCH, R.G. (1988) IgA:IgM and IgA:IgA hybrid hybridomas secrete heteropolymeric immunoglobulins that are polyvalent and bispecific. *J. Immunol.* **140,** 558.

VAN DIJK, J., WARNAAR, S.O., VAN EENDENBURG, J.D., THIENPONT, M., BRAAKMAN, E., BOOT, J.H., FLEUREN, G.J. & BOLHUIS, R.L. (1989) Induction of tumor-cell lysis by bi-specific monoclonal antibodies recognizing renal-cell carcinoma and CD3 antigen. *Int. J. Cancer*, **43,** 344.

VYAKARNAM, A., STRANGEWAYS, A.L., GLOVER, R.E. & LACHMANN, P.J. (1988) Cytolysis by cloned helper T cells: induction by specific antigen or by anti-CD3 hybrid antibodies. *Scand. J. Immunol.* **27,** 635.

WARD, E.S., GUSSOW, D., GRIFFITHS, A.D., JONES, P.T. & WINTER, G. (1989) Binding activities of a repertoire of single immunoglobulin variable domains secreted from *Escherichia coli*. *Nature*, **341,** 544.

WEBB, K.S., WARE, J.L., PARKS, S.F., WALTHER, P.J. & PAULSON, D.F. (1985) Evidence for a novel hybrid immunotoxin recognizing ricin A-chain by one antigen-combining site and a prostate-restricted antigen by the remaining antigen-combining site: potential for immunotherapy. *Cancer Treatment Rep.* **69,** 663.

WEBB, K.S., POULTON, S.H., LIBERMAN, S.N. & WALTHER, P.J. (1989) Rationale for immunotoxin therapy of metastatic prostate carcinoma formatted as a multi-stage delivery system. *J. Urol.* **142,** 425.

WONG, J.T. & COLVIN, R.B. (1987) Bi-specific monoclonal antibodies: selective binding and complement fixation to cells that express two different surface antigens. *J. Immunol.* **139,** 1369.

ZIMMERMAN, B. & GREY, H.M. (1971) Noncovalent interaction between immunoglobulin (Ig) polypeptide chains. II. Absence of hybrid molecule formation between Ig classes. *J. Immunol.* **107,** 1788.